REMARKS

Claims 1-25 are pending in this application and have been rejected. A supplemental information disclosure statement accompanies this response. Applicants request that the Office consider the contents of the cited references therein.

In addition, the Office has objected to claim 16 because it is asserted that this claim employs a trademark. This claim has been canceled, rendering the rejection moot. Applicants have amended the specification to account for trademarks where they occur and to correct obvious typographical errors. Applicants request the rejection of the application on the basis of the noted informality be withdrawn.

Claims 8, 11 and 16-19 have been rejected under 35 U.S.C. § 112, second paragraph as indefinite, with particular reference to the phrases "exons 5 to 9 of the p53 gene" in claim 8, "having an average size of at least about 20kb" in claim 11 and "(Tag/GB-D)" in claim 16. Claims 8 and 11 have been amended to clarify the meaning of the claims and claim 16 has been canceled. Applicants therefore submit that the claims fully comply with 35 U.S.C. § 112, second paragraph and request that the rejections of claims 8, 11 and 16-19 on this basis be withdrawn. Support for the claim amendments can be found in paragraphs 21, 29, 40 and 43 of the specification, as well as the original claims.

Claims 1 and 7 have been rejected under 35 U.S.C. §102(b) as anticipated by Sands et al., which is cited as teaching a method for determining mutation load by identifying a somatic cell with accumulated p53 levels, amplifying the cell's p53 gene DNA and determining the frequency of mutation in the amplified DNA. The Office also indicates that the reference shows that amplification may be performed in the presence of bovine serum albumin.

For a reference to anticipate a claim, it must disclose within its four corners, each and every element of that claim. M.P.E.P. §

2131. Applicants therefore traverse this rejection. Claim 1 has been amended to clarify the invention intended to be claimed. The invention involves identifying a somatic cell in a tissue sample that contains accumulated p53 and amplifying the DNA <u>of that cell</u> by PCR. The amplified DNA of the identified cell is then analyzed to determine mutation frequency.

The Sands et al. reference describes a method for testing mutation frequency in mice bearing λ shuttle vector Big Blue (see pp. 8571-8518). The methods depend on expression of blue phenotype (see p8518), and therefore can be used only in cells with this endogenous marker; these methods are not suitable for use in humans. Further, genomic DNA was not isolated <u>from a cell which had been identified</u> as having accumulated p53 levels. First the cells were <u>genotyped</u> as either p53(-/-) or p53(+/+), passaged four times and then plated twice to expand the cells (see p8518). Consequently it is not known whether these cells, or any particular cell in this artificially created cell line had accumulated p53 gene product. Second, DNA from the originally genotyped cells was not amplified by PCR. These cells were passaged and expanded before DNA isolation from large groups of cells, in vitro packaging preabsorption to E. coli and plating (see p85158). No PCR amplification was performed at all, and certainly not on DNA of a cell identified as containing accumulated p53. Third, Blue plaques containing mutant lacI genes were chosen for analysis to compare cells having a p53 genes to cells lacking a p53 gene. The mutational status of the p53 gene or accumulation of p53 is not the basis for identifying any cells.

Sands et al. therefore does not disclose (1) identifying a somatic cell in a tissue sample that contains accumulated levels of p53, (2) amplifying by PCR DNA of <u>said identified somatic cell</u>, or (3) determining the frequency or nature of mutations in <u>said amplified DNA</u>. Applicants submit that the Office can not make out a case of anticipation by Sands et al. because this reference fails

to disclose at least one element of the rejected claims, and request that the rejection of claims 1 and 7 under 35 U.S.C. § 102(b) be withdrawn.

Claims 1, 8-11 and 15-16 have been rejected under 35 U.S.C. § 102(e) as anticipated by Diamandis et al. The Office contends that Diamandis et al. teach a method for determining mutation load by identifying a somatic cell which has accumulated p53 levels, amplifying p53 gene DNA of this cell and determining the frequency of mutation in the amplified DNA using two different DNA polymerases to amplify DNA of 1kb, 2kb or 20kb from exons 5-9 of p53.

Applicants traverse this rejection. Diamandis et al. describe a hierarchical system of testing patient samples for the presence of p53 mutation which involves (1) immunological assays for p53 mutation (2) DNA fragment analysis of different aliquots of samples <u>negative</u> for p53 mutation in step (1), and (3) DNA sequence analysis of samples <u>negative</u> for p53 mutation in step (2). No somatic cell was identified as containing accumulated levels of p53 by Diamandis et al. Rather, a test for anti-p53 antibodies in patient serum was conducted (example 1; cols. 8-10). This test does not and cannot identify cells having elevated p53. In level 2 of the hierarchy, DNA is prepared from a patient blood sample in which no blood cells have been identified as having accumulated p53 levels (see cols. 10-11). Pooled DNA from many blood cells in the sample is amplified in several multiplex PCRs for the purpose of fragment length analysis of p53. In level 3 of the hierarchy, a second and separate DNA sequence analysis is performed using the same type of pooled DNA sample from a mixture of blood cells as for Level 2. Thus, only an "average" sequence analysis of the DNA in this passage cell line can be obtained, which is not equivalent to the method claimed here.

In no case is any cell identified as containing accumulated levels of p53, nor is the DNA of any identified cell amplified.

Diamandis et al. do not teach that the DNA of any identified cell somatic cell that contains accumulated levels of p53 could or should be amplified. The Diamandis et al. reference therefore lacks a disclosure of at least one element of the invention claimed here and cannot form the basis of an anticipation rejection. Applicants therefore request withdrawal of the rejection of claims 1, 8-11 and 15-16 over Diamandis et al.

Claims 2-3, 5-6, 12-14 and 20-21 have been rejected under 35 U.S.C. § 103(a) as obvious over Diamandis et al. in view of Taylor et al. The many deficiencies of the disclosures of Diamandis et al. are discussed above. In summary, Diamandis et al. do not teach or even suggest that a cell with accumulated p53 levels can or should be identified, and do not teach or suggest amplification of the DNA of such an identified cell. As the Office notes, Diamandis et al. also do not teach paraffin-embedded tissue sectioning and immunohistochemical staining for p53.

Taylor et al. is cited for teaching obtaining a single somatic cell by microdissection from an ethanol-fixed, paraffin-embedded tissue section of a sample from a cancer patient and identification of a cell staining for p53 by immunohistochemistry. The Office reasons that one of ordinary skill would be motivated to apply the above methods of Taylor et al. to the Diamandis et al. methods for determining mutation load in order to enhance sensitivity of immunohistochemical methods and reduce false negative results.

Applicants traverse this rejection. Although it may have been desirable to perform both an immunohistochemical test and a DNA test on samples from cancer patients to increase the accuracy of cancer diagnosis and prognosis, there is nothing in the cited art that would lead the person of ordinary skill in the art of the invention which is claimed here.

To make out a prima facie case of obviousness, the Office must meet three criteria: the cited prior art references must teach or suggest each and every claim limitation, the prior art must contain

a suggestion or motivation to modify the cited reference(s) to achieve the invention of the claims and there must be a reasonable expectation of success. M.P.E.P. § 2143. Applicants submit that the Office cannot make out a prima facie case of obviousness with respect to this invention.

The Diamandis et al. reference does not teach or suggest the amplification and study of DNA from a cell containing accumulated p53 levels, as discussed above. There is no teaching or even a hint that would allow the person of skill in the art to achieve amplification and study of an identified cell using the methods disclosed by Diamandis et al. The methods, as discussed above, involve preparation of DNA from groups of untested cells in a blood sample, followed by multiplex PCR to compare p53 fragment lengths. This method would not achieve the result of the invention claimed here. Furthermore, there is no motivation in the reference to modify the teachings to be able to study DNA in an identified somatic cell even if the methods taught would allow it.

Taylor et al. teach methods for performing immunohistochemistry on paraffin-embedded sections, including antigen retrieval and staining for p53, however these teachings do not make up for the deficiencies of Diamandis et al. Nothing in this reference provides the disclosures necessary to be able to modify the prior art p53 fragment length analysis of pooled DNA from a patient blood sample of Diamandis et al. to the amplification and analysis of DNA from an identified somatic cell. Taylor et al. only describes a method of unmasking antigens in tissue sections to improve sensitivity of immunohistochemistry. This is a method of identifying cells having a particular antigen, such as p53. It does not even suggest that a cell identified by these methods could or should be studied by amplification of its DNA and determination of the frequency or nature of mutations in that amplified DNA, much less provide methods by which this could be achieved. Therefore nothing in the Diamandis et al. or Taylor et al. references, alone

or in combination, teaches or even suggests each and every element of the method claimed here. These references do not teach or suggest that it is possible or even desirable to amplify the DNA of a cell that is identified as containing accumulated levels of p53, nor do they teach or suggest that this DNA should be studied to determine the frequency or nature of mutations in it.

Moreover, nothing in either reference provides a motivation to combine identification of a p53 accumulating cell with DNA amplification, since the methods taught by these references could not even accomplish such a method. Even if one were to recognize that cells that contain accumulated levels of p53 should be studied by the methods of Diamandis et al. (which Applicants maintain would not be recognized by one of skill in the art due to the incompatibility of the methods) this could not be achieved using the disclosed methods. The most that could be achieved by combining the teachings of Diamandis et al. and Taylor et al. is a method for amplifying pooled DNA from blood samples of patients who have been identified as having cells that contain accumulated levels of p53 in paraffin-embedded sections and then testing the DNA from blood cells for p53 fragment length. The blood of Diamandis et al. could not be fixed in ethanol, paraffin-embedded and sectioned, and the positive cells of the Taylor et al. tissue section could not be amplified by the Diamandis et al. multiplex PCR. The two methods would not be recognized by the skilled person as usable together. Therefore, there is no motivation to combine these methods with any chance of success, much less a reasonable chance.

Applicants respectfully submit that the Office cannot meet the three criteria listed above because (1) the combined references do not teach or even suggest that the DNA of an identified cell could be amplified and studied, a required element of claim 1 and hence all the rejected claims, (2) the combined references do not show or even suggest how the methods could be combined to achieve

amplification of an identified cell's DNA, nor that this would even be desirable, or (3) any reasonable chance that the present invention could be achieved successfully. Applicants therefore request that the rejection of claims 2-3, 5-6, 12-14 and 20-21 be withdrawn.

Claims 22-24 have been rejected under 35 U.S.C. § 103(a) as obvious over Diamandis et al. in view of Taylor et al. and Link et al. The deficiencies of the disclosures of Diamandis et al. and Taylor et al. have been discussed at length above. The same reasoning applies here. Neither of these references teaches or even suggests that DNA of a cell identified as containing accumulated levels of p53 could or should be amplified and studied as claimed here or provided any motivation which shows that this should be attempted, how it could be done or why success would be reasonable to expect.

The Link et al. reference is cited by the Office for its teachings concerning cytotoxic or gene therapy treatment for cancer. These teachings are completely unrelated to the invention claimed here. The invention here is not a method of killing neoplastic cells, as is implied by the Office Action at page 7, lines 1-4. Nor does the claimed method involve performing any type of cancer treatment. Claims 22-24 only specify that the somatic cell upon which the method is performed is obtained from a patient currently receiving the recited known cancer treatments.

The disclosures of Link et al. therefore do not make up for the complete lack of teaching or suggestion in the Diamandis et al. and Taylor et al. references discussed above because they do not provide a method of amplifying DNA in an identified cell and determination of the frequency or nature of mutations in the amplified DNA of that cell, whether the source of that cell is a cancer patient currently receiving cancer treatment or not. Applicants submit that the combination of Diamandis et al., Taylor et al. and Link et al. do not and can not result in a method which

achieves what the invention claimed here can and does achieve. Applicants therefore refer the Office to the discussions regarding Diamandis et al. and Taylor et al. above and request reconsideration and withdrawal of the rejection of claims 22-24 under 35 U.S.C. § 103(a).

Claim 4 has been rejected under 35 U.S.C. § 103(a) as obvious over Diamandis et al. in view of Taylor et al. and Havemann et al. The deficiencies of the disclosures of Diamandis et al. and Taylor et al. have been discussed at length above. Applicants refer the Office to these discussions. The teachings fairly derived from Havemann et al. do not make up for these glaring deficiencies. Havemann et al. is cited by the Office as teaching "the p53 regulated proteins mdm2 [ ] and vEGF [ ]." The Office concludes that the person of ordinary skill in the art would have been motivated to apply the p53-regulated proteins mdm2 and vEGF to the combined method of Diamandis et al. and Taylor et al "in order to isolate somatic cells."

The disclosures of Havemann et al. are directed to methods of culturing mononuclear cells useful, ultimately, for gene therapy methods after transfection with a desirable gene. The protein vEGF is disclosed in the context of providing a growth factor to cultured cells, as a promoter for gene expression or as an example of a ligand, not as a method, along with accumulation of p53 levels, for identifying a cell for DNA amplification and analysis (see Havemann et al. ¶¶ 37, 73-74, 105, 159, 241, and the Examples). The protein mdm2 is disclosed in the context of a method for immortalizing cells by transforming with a protein that inactivates a suppressor gene product. Nothing in the disclosures of Havemann et al. teaches, suggests or even hints that these two proteins might be used to identify a somatic cell suitable for DNA amplification and study. Neither does the Havemann et al. reference disclose or suggest anything from which the person of ordinary skill in the art could infer that a cell so identified

could or should be studied by the method claimed here, or even that if one attempted to do so the methods of Diamandis et al. and Taylor et al. could be combined with a reasonable chance of successfully achieving the invention claimed here. In short, Havemann et al. cannot make up for the deficiencies in the disclosure of the primary references as discussed above. Applicants refer the Office to this discussion and request that the rejection of claim 4 under 35 U.S.C. § 103(a) be withdrawn.

Claims 17 and 18 have been rejected under 35 U.S.C. § 103(a) as obvious over Diamandis et al. in view of three additional references: Allalunis-Turner et al., Rozemuller et al. and Newton. Claim 17 is cancelled herein, rendering its rejection moot.

Allalunis-Turner et al. is cited by the Office as teaching a 3407 bp nucleotide sequence that shares a 30 bp region with SEQ ID NO:1 starting at locus 1483. Likewise, Rozemuller et al. is cited as disclosing all exons of the p53 gene and a 20303 bp nucleotide sequence that shares a 30 bp region with SEQ ID NO:2 starting at locus 14862. The Office refers to a STIC Report which was not included in the materials provided with the Office Action.

The Office has conceded that Diamandis et al. does not teach a 30 nucleotide base primer matching SEQ ID NO: 1 and a 30 nucleotide base primer matching SEQ ID NO:2. Nor do Allalunis-Turner et al., nor Rozemuller et al., nor Newton. Applicants have been able to locate a complete copy of the Allalunis-Turner et al. reference (Radiat. Res. 134:349-354, 1993) cited by the Office and are submitting this reference as part of the information disclosure statement filed with this response. This reference relates to two cell lines isolated from a tumor biopsy sample which are tested for sensitivity to radiation and drug treatments. Nowhere in this reference is a 3407 bp nucleotide sequence disclosed, or, in fact, any nucleotide sequence. The authors of Allalunis-Turner et al. do not even mention a specific nucleotide sequence that Applicants can discover and in particular do not mention primers such as those

recited in the rejected claims 17 and 18. There is nothing in this reference that even suggests the primers of claims 17 and 18, or any method for determining mutation load as claimed in the present application. Applicants therefore submit that Allalunis-Turner et al. is completely inapposite and cannot form a basis for any rejection here and invite the Office to review the complete reference for its fair teachings.

Rozemuller et al. disclose a series of primers used for sequence-based mutation analysis of p53 and also disclose corrections to a previously published genomic sequence as well as identified polymorphisms in p53. Applicants have been able to locate a complete copy of this reference and are submitting this reference as part of an information disclosure statement filed with this response. Applicants request that the Office review the cited reference for its fair teachings.

None of the primers listed in Table 1 of Rozemuller et al. contain the nucleotide sequences of SEQ ID NOS:1 or 2. Moreover, nothing in Rozemuller et al. teaches or even remotely suggests primers for the method claimed in this application. Therefore, the teachings and fair suggestions contained in Rozemuller et al. do not make up for the deficiencies of Diamandis et al. which, as discussed above, do not teach or suggest a method which is capable of amplifying DNA of a cell identified to contain accumulated p53. Nothing in Rozemuller et al. would allow the skilled person to modify the Diamandis et al. methods to achieve the present invention, even if one accepts the Office's assertions that Rozemuller et al. do disclose the entire sequence of the p53 gene. Knowing this sequence does not enable the presently claimed method using the techniques of Diamandis et al. Moreover, neither reference contains any motivation to combine the teachings to achieve this, even if it could be done, and certainly not with any reasonable expectation of success.

The Newton reference relates to helpful hints for primer design given a specific list of techniques provided in Table 1 (p. 55), with the goal of achieving maximized specificity and efficiency. The reference does not mention that any particular technique or hint should be used with respect to the type of method in the present application (amplifying DNA of a cell identified to contain accumulated p53) and therefore cannot provide any motivation to achieve the presently claimed method from the disclosures of Diamandis et al. For multiplex PCR, the technique used by the Diamandis et al. authors, Newton recommends paying attention to the following parameters: (1) primer positions with respect to target coding in non-coding sequence; (2) the melting temperature of the primers; and (3) complementarity between the ends of primers. Applicants submit that attention to these parameters when modifying the multiplex PCR methods of Diamandis et al. on DNA samples obtained from large groups of blood cells will not result in the invention achieved here. Therefore, the disclosures and fair suggestions of these references, even if the complete sequence of the p53 gene is deemed to be known, do not contain each and every element of the rejected claim, cannot be modified to attain the claimed invention, and have no reasonable chance of success.

Applicants therefore submit that the rejection of claims 17 and 18 under 35 U.S.C. § 103(a) is not proper and should be withdrawn.

Claim 19 has been rejected under 35 U.S.C. § 103(a) as obvious over Diamandis et al. in view of three additional references: Allalunis-Turner et al., Felix et al. and Newton. Allalunis-Turner et al. and Newton have been discussed above. Applicants have explained why these references alone or in combination with Diamandis et al. cannot form a proper basis for an obviousness rejection. Applicants have been able to locate a copy of the Felix et al. reference and are submitting this reference in an information disclosure statement filed with this response. Felix et al. discuss p53 gene mutations present in childhood acute

lymphoblastic leukemia. DNA was prepared from the blood tumor "as described [in prior publications]." No indication whatsoever is given that the DNA is obtained from a cell identified as containing accumulated p53. The DNA was probed with three riboprobes using a PCR/SSCP method to screen for point mutations.

Applicants assume that the 133 nucleotide sequence referred to by the Office in making the claim rejection is that described on page 642 and in Figure 2 of Felix et al. (nt 196-328 of intron 9). This sequence does not teach or suggest the 33 nucleotide sequence of SEQ ID NO:3. The Felix et al. sequence, beginning on the third line of the insert in Figure 2, discloses a sequence which contains the complement of SEQ ID NO:3. There is nothing in the Felix et al. reference, however, which teaches, suggests, or even hints that the indicated 33 base portion of the disclosed sequence should be carved out of the disclosed sequence, converted to the complement and used as a primer to amplify DNA from a cell identified as containing accumulated p53. The sequence is disclosed only as a gene area that is alternatively spliced in certain patients. Felix et al. therefore adds nothing to the disclosures of the other cited references in terms of developing the present invention from the methods of Diamandis et al. There is nothing that would enable the Diamandis et al. methods to be modified and adapted to achieve the present invention, no motivation to even attempt to do so, and no reasonable expectation of success.

Applicants therefore submit that the Office cannot make out a case of *prima facie* obviousness against claim 19, here rejected, and request that the rejection be withdrawn.

Claim 25 has been rejected under 35 U.S.C. § 103(a) as obvious over Diamandis et al. in view of Murphy and Newton. The disclosures of Diamandis et al. and Newton has been discussed above. Applicants have explained why the helpful hints concerning primer design would not assist in modifying the multiplex PCR methods on DNA from blood samples to achieve a method involving

amplifying DNA from a cell identified as containing accumulated p53. Applicants refer the Office to the discussions above in this regard.

The disclosures of Murphy in Example 6, p. 46, relate to primers for sequencing of mutations by PCR and include primer 5F, which contains the 24 nucleotides of SEQ ID NO:5 of claim 25. However, knowing the sequence of this primer would not assist the person of skill in the art to modify the teachings of Diamandis et al. and Newton with regard to multiplex PCR of DNA from a pool of blood cells to attain the claimed method. The methods simply cannot be used to amplify DNA from a cell identified as containing accumulated p53 without a great deal of further information and technical know-how which is provided only in the disclosures of the present specification. Further, there is no motivation in Murphy or any of the cited prior art to truncate the Murphy primer for use in such a method. Even if the primer of Murphy was truncated, it could not be used according to the methods of Diamandis et al. as informed by Newton to achieve the methods claimed here.

Applicants submit that the Office cannot make out a *prima facie* case of obviousness for claim 25 based on the references cited here because the references lack an essential teaching or suggestion of at least one claim element and the motivation to modify the teachings. Applicants therefore request that the rejection of claim 25 under 35 U.S.C. § 103(a) be withdrawn.

In summary, nothing in the prior art teaches or suggests a method whereby a somatic cell is identified as containing accumulated levels of p53, DNA of that identified cell is amplified by PCR and the frequency or nature of mutations in that amplified DNA is determined. The claims thus are patentable over the cited prior art.

Applicants have amended the claims to more clearly identify what is the invention intended to be claimed, to remove abbreviated terms, to add proper Markush terminology and to remove multiple

dependencies and to correct other minor claim informalities. Applicants submit that the claims are now in condition for allowance.

In view of the foregoing amendments and remarks, Applicants request reconsideration of the application and withdrawal of the pending rejections.

<table>
<tr><td colspan="6">RESPECTFULLY SUBMITTED,</td></tr>
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1954-360.AMD

Mark-up Version of Amended Specification:

[0008] To identify individuals who are predisposed to elevated spontaneous mutations or who have had previous carcingen exposure, it is advantageous to use the smallest amount of PCR template that could result in an accurate picture of mutation load. HotStart™ (HOTSTART) PCR, which helps to prevent the formation of primer dimer, permits amplification of more dilute template. Using this technique, a 140 bp PCR segment was amplified from an extracted single DNA template using 60 cycles. Vogelstein, B., Kinzler, K.W., "Digital PCR", Proc. Natl. Acad. Sci. U.S.A. 96:9236-9241 (1999). This technique involves the fewest number of PCR cycles required for dependable results reported to date.

[0018] Ethanol is a preferred tissue fixative[,] because it precipitates antigens and does not cause DNA crosslinking, as does formalin. Following ethanol fixation, tissue is embedded in paraffin and sliced in thin sections by standard procedures. Routine pathology sections conventionally are between 4 µm and 5 µm in diameter. When using this diameter, tissue sections consist not only of undamaged sections, but also contain some damaged nuclei, resulting in allele dropout. To enhance the possibility of obtaining undamaged nuclei and reducing the risk of dissecting damaged cell nuclei, large sections, e.g., about 6 µm or greater are preferred. Steam heating using an EDTA buffer has been found to yield reliable immunohistochemical staining and intact DNA. Taylor, C.R., Shi. S.R., Cote, R.J., "Antigen retrieval for immunohistochemistry status and need for greater standardization", Applied Immunohistochemistry 4:144-146 (1996). Suitable conditions include a buffer containing 1mM EDTA (pH 8.0) at 96 to 100° C and heating for 5 minutes. Alternatively, steam heating can be performed using 20 mM HEPES/1mM EDTA buffer (pH 8.1) with a pK much less affected by high temperature. Using these process

enhancements, the size of the single stranded DNA has been determined to have an average length of 20 kb.

[0019] Cells that accumulate p53 or have altered levels of a protein which is the product of a gene whose expression is regulated by p53 may be identified by immunohistochemical staining. Monoclonal antibodies that may be used for this purpose are available commercially. See Examples, infra. Because mutant p53 accumulates in cells, staining for this protein is useful for identifying cells in which mutations have occurred. The p53 protein regulates the expression of a number of other genes, including PCNA, mdm2 and vEGF. Thus the levels of the proteins that are the products of these genes often are altered in cells containing mutant p53. For example, p53 down regulates PCNA, and [mutatnt] <u>mutant</u> p53 may result in accumulated levels of PCNA in cells. The levels of other proteins under p53 control may be increased or decreased, depending on the mechanism of the control. The use of the levels of expression of one or more of such secondary proteins assists in [diffrentiation] <u>differentiation</u> between cells having enhanced levels of wt p53 resulting from natural physiological induction and the cells of interest having accumulated levels of mutant p53.

[0021] Mutation analysis, including determination of mutation load, advantageously is determined by amplification and analysis of DNA from a single cell. The DNA is amplified by any procedure that efficiently reproduces DNA from the low template concentrations obtained from a single cell. A preferred amplification procedure, referred to herein as "Stimulated PCR," has been found to yield sufficient DNA for sequence analysis using as [fews] <u>few</u> as 40 cycles of amplification. This PCR process differs from known processes in that it substantially reduces the threshold effect of the template concentration on PCR efficiency. Additional

advantages of Stimulated-PCR may include: inhibition of absorbance of the template to the tube surface; protection against minimal DNAase activity; addition of false priming sites for spurious extensions; activation by binding DNA polymerase; or direct stimulation of extension by DNA polymerase. Stimulated PCR is described in detail in the Examples, infra. In general, the technique is characterized by the use of a combination of a Taq polymerase High Fidelity™ and Taq DNA polymerase and by the incorporation of mouse genomic DNA having an average size of more than about 20 kb. The addition of mouse genomic DNA allows a wider range of annealing temperatures, a wider range of primer concentrations, less primer dimer formation, and higher product yield. Similar effects were observed by adding or supplementing bovine serum albumin (BSA), probably because BSA protein assists in keeping DNA polymerases in active forms.

[0022] Changes in the use of High Fidelity™ enzymes, commercially available and utilized by those of skill in the art, improve amplification yields. The highest yields are found when the High Fidelity™ enzymes are used in higher amounts than those typically used, e.g., at about 4-fold the amount recommended by the manufacturer (2.5 U Taq/GB-D DNA polymerases per 25 µl of reaction). Additionally, mixing 1 U of [Platinum] <u>PLATINUM</u> Taq with 1 U of the High Fidelity™ enzymes, which increases the unit ratio of Taq to GB-D by 2 fold, behaves better than the High Fidelity™ enzymes alone, indicating that not only the total units, but also the relative ratios of the enzymes are important. Another improvement that may be used to increase the fidelity of the amplification and to minimize primer-dimer formation is the incorporation of a Taq antibody to inactivate Taq DNA polymerase at room temperature. This improvement is used in so-called [HotStart] <u>HOTSTART</u> PCR. In the present method, it has been effective in preventing primer dimer formation using 40 to 45 cycles.

[0025] b. T_m of the primer was estimated by the nearest neighbor method at 50 mM [Kcl] KCl and 250 pM DNA and T_m of the PCR segment was estimated by the formula of Wetmur: $T_m^{product} = 81.5 + 16.6 \log[K^+= 0.05\ M] + 0.41\ (\%G + \%C) =675/\text{length}$.

[0036] The PCNA antibody (Ab-1 monoclonal mouse IgG antibody) (Oncogene [Calbiocaem] Calbiochem) was used in a concentration of 1:4000; the p53 antibody (mouse monoclonal antibody DO7) (Novocastra) was used in a concentration of 1:100. The tissue sections were double stained immunohistochemically for p53 and PCNA. Cells testing positive showed p53 positive nuclear staining (bright red), PCNA positive cytoplasmatic staining (light brown) or both PCNA and p53 positive staining (reddish brown).

[0037] The single cells were manually microdissected using an inverted microscope (Nikon TMS) and a mechanical micromanipulation system (Sutter Instruments). A tungsten needle was manipulated through a joystick. The microdissected cell was then picked up manually with a new 27 G ½" needle, and transferred into a 0.2mL PCR tube containing 5µl digestion buffer: #3 High Fidelity™ buffer without Magnesium, 2 mg/ml Proteinase K (Qiagen), 3% Tween-20 detergent and 0.2mM EDTA (pH 8.0). The single cell was digested at 50°C for 16 hr and after the digestion, Proteinase K was inactivated at 90 °C for 10 minutes. This single cell was then amplified by Stimulated PCR as set forth below in Example II.

[0039] In order to detect mutations in the single cell chosen for microdissection isolation from the paraffin-embedded tissue, the single cell was subjected to the Stimulated PCR technique. In preparing for the Stimulated-PCR used to amplify the single cell mutations, primer selection is important. Here, all primers were designed and analyzed with Oligo 5 software (National Biosciences). T^m of the primer was estimated by the nearest neighbor method at 50

mM KCL and 250 pM DNA and Tm of the PCR segment was estimated by the formula of Wetmur: $T^{m}_{product} = 81.5 + 16.6 \log[K^{+}=0.05M] + 0.41(\%G + \%C) - 675/length$. Wetmur, J.G., "DNA probes: Applications of the Principles of Nucleic Acid Hybridization", Critical Rev. in Biochem and Mol Biol. 26:227-259 (1991), the disclosure of which is incorporated herein by reference. The criteria for specificity included high-specificity with low base-pairing stability at the 3′ end, no primer-dimer or hairpin formation more than 3 bases at the 3′ end, no homo- or repeat-sequence at the 3′ end, and no false priming site more than 7 bases at the 3′ end for any strand and any segment. The primer also had no false priming site on the mouse p53 gene to generate spurious products. The primers used are shown in Table 1, above. For Stimulated PCR, primer GCCGTCTTCCAGTTGCTTTATCTGTTCACT (SEQ. ID. NO. 1) was used in conjunction with either CCTGATGGCAAATGCCCCAATTGCAGGTAA (SEQ. ID. NO. 2) [and] <u>or</u> GTCAAGTAGCATCTGTATCAGGCAAAGTCATAG (SEQ. ID. NO. 3). The results of a 2 kb region of the p53 gene amplification with 0.6 µl of p53(12983)30D (SEQ. ID. NO. 1) and p53(15036)33U (SEQ. ID. NO. 3) from genomic DNA of twelve single microdissected cells for 40 cycles are shown in Figure 2.

[0040] The PCR mixtures contained a total volume of 25 µl: human genomic DNA from a dissected single cell; #3 Expanded High Fidelity buffer (Boehringer Mannheim); 3.5 mM $MgCl_2$; 500 µM of each dNTP; 2% DMSO; 0.2 to 0.6 µM of each of primers; a mixture of 1.25U of [Platinum] <u>PLATINUM</u> Taq DNA polymerase High Fidelity (Taq/GB-D)/1.25U of [Platinum] <u>PLATINUM</u> Taq DNA polymerase (GIBCO BRL); 5 µg of BSA and 25 ng of mouse genomic DNA with the average size more than 20 kb. The cycling conditions included denaturation at 92 °C for 12 seconds, annealing at 60 °C for 20 seconds, and elongation at 68 °C for 2 minutes for 40 or 45 cycles with a Perkin Elmer GeneAmp PCR system 9700. An additional 20 seconds of denaturation time proceeded the first cycle. Two to 4 µl of the PCR product was

electrophoresed through a standard 1% agarose gel and then the gel was stained with ethidium bromide for UV photography by a CCD camera (Bio-Rad Gel Doc™ 1000) and Multi-Analyst™ software (version 1.1). Another nested or half nested PCR was performed for 12 to 15 cycles to obtain more product. For this additional half-nested PCR, primers CCTGATGGCAAATGCCCCAATTGCAGGTAA (SEQ. ID. NO. 2) and TGTTCACTTGTGCCCTGACTTTCAACTCTG [SEQ. ID NO. 4] were used.

[0041] The PCR product was purified for two rounds using Microcon® 100 (Amicon) to remove the unincorporated primer and primer dimers. Standard sequence analysis was performed using ABI 377 fluorescence sequencer and BigDye™ terminator chemistry with [AmpliTaq] AMPLITAQ FS DNA polymerase (PE Applied Biosystem). The primers used during the sequencing process were: TGCCCTGACTTTCAACTCTGTCTC (SEQ. ID. NO. 5); AGGGTCCCCAGGCCTCTGAT (SEQ. ID. NO. 6); GGCCACTGACAACCACCCTTAA (SEQ. ID. NO. 7); AGGTCTCCCCAAGGCGCACT (SEQ. ID. NO. 8); GGGGCACAGCAGGCCAGTGT (SEQ. ID. NO. 9); GGAGAGACCGGCGCACAGA (SEQ. ID. NO. 10); and CGGCATTTTGAGTGTTAGACTGGA (SEQ. ID. NO. 11).

[0044] a. All of the above were identified from 15 normal nontumorous colon cells with *p53* and PCNA double straining. The missense mutations are either at a conservative site [r] or are found in the *p53* mutation database (http://www.iarc.fr/P53/index.html) Mutations were also identified in other normal cells of breast, lung, kidney and gallbladder.

Mark-up Version of Amended Claims:

1. (Amended) A method for determining mutation load which comprises identifying a somatic cell that contains accumulated levels of p53, amplifying by PCR DNA of [the p53 gene from such] said identified somatic cell and determining the frequency or nature of mutations in [the] said amplified DNA.

2. (Amended) The method of claim 1, in which [the] said somatic cell [that is identified] also contains altered levels of a protein [selected from the group consisting of PCNA and other proteins] that [are] is regulated by p53.

3. (Amended) The method of claim 2, wherein [the] said protein regulated by p53 is [PCNA} proliferating cell nuclear antigen.

4. (Amended) The method of claim 2, wherein [the] said protein regulated by p53 is [mdm2 or vEGF] selected from the group consisting of murine double minute chromosome clone number 2 and vascular endothelial growth factor.

5. (Amended) The method of claim 1, in which [the] said somatic cell is identified by immunohistochemical staining for p53.

6. (Amended) The method of claim 2, in which [the] said somatic cell is identified by immunohistochemical staining for p53 and [a] for said protein [selected from the group consisting of PCNA and other proteins that are] regulated by p53.

7. (Amended) The method of claim 1 [or 2], in which [the] said amplification is conducted in the presence of a compound selected from the group consisting of mouse DNA [or], bovine serum albumin [or] and both mouse DNA and bovine serum albumin.

8. (Amended) The method of claim [1 or 2] 26, wherein [the] said DNA [that is amplified is from] of the human p53 gene contains a segment in the human p53 gene spanning exons 5 to 9 [of the p53 gene].

9. (Amended) The method of claim [1 or 2] 8, wherein [the] said DNA [that is amplified] is at least 1 kb in size.

10. (Amended) The method of claim [1 or 2] 8, wherein [the] said DNA [that is amplified] is at least 2 kb in size.

11. (Amended) The method of claim [1 or 2] 7, in which [the amplification is conducted in presence of] said mouse DNA [having] has an average size of at least about 20 kb.

12. (Amended) The method of claim 1 [or 2], in which the method is performed on a single somatic cell which is obtained by microdissection from a paraffin-embedded tissue section.

13. (Amended) The method of claim 12, in which [the] said tissue section is fixed with ethanol.

14. (Amended) The method of claim 12 in which [the] said tissue section is subjected to steam heating in the presence of EDTA [to facilitate unmasking of antigen site].

15. (Amended) The method of claim 1 [or 2], in which the amplification step [utilizes] is performed using two different DNA polymerases.

18. (Amended) The method of claim [17,] 11, in which [further comprises use of] amplification is performed using primers [of the sequence] GCCGTCTTCCAGTTGCTTTATCTGTTCACT (SEQ. ID. NO. 1) and CCTGATGGCAAATGCCCCAATTGCAGGTAA (SEQ. ID. NO. 2).

19. (Amended) The method of claim [17,] <u>11, in</u> which [further comprises use of] <u>amplification is performed using</u> primers [of the sequence] GCCGTCTTCCAGTTGCTTTATCTGTTCACT (SEQ. ID. NO. 1) and GTCAAGTAGCATCTGTATCAGGCAAAGTCATAG (SEQ. ID. NO. 3).

25. (Amended) The method of claim 1 [or 2], in which the frequency or nature of mutations is determined by sequence analysis [which utilizes one or more] <u>using a primer selected from the group consisting</u> of TGCCCTGACTTTCAACTCTGTCTC (SEQ. ID. NO. 5), AGGGTCCCCAGGCCTCTGAT (SEQ. ID. NO. 6), GGCCACTGACAACCACCCTTAA (SEQ. ID. NO. 7), AGGTCTCCCCAAGGCGCACT (SEQ. ID. NO. 8), GGGGCACAGCAGGCCAGTGT (SEQ. ID. NO. 9), GGAGAGACCGGCGCACAGA (SEQ. ID. NO. 10)[,or] <u>and</u> CGGCATTTTGAGTGTTAGACTGGA (SEQ. ID. NO. 11)[as primers].